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REVIEW ARTICLE

Steps to Translate Preconditioning from Basic Research to the Clinic

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Abstract Efforts to treat cardiovascular and cerebrovascular diseases often focus on the mitigation of ischemia–reperfusion (I/R) injury. Many treatments or "preconditioners" are known to provide substantial protection against I/R injury when administered prior to the event. Brief periods of ischemia itself have been validated as a means to achieve neuroprotection in many experimental disease settings, in multiple organ systems, and in multiple species suggesting a common pathway leading to tolerance. In addition, pharmacological agents that act as potent preconditioners have been described. Experimental induction of neuroprotection using these various preconditioning paradigms has provided a unique window into the brain's endogenous protective mechanisms. Moreover, preconditioning agents themselves hold significant promise as clinical-stage therapies for prevention of I/R injury. The aim of this article is to explore several key steps involved in the preclinical validation of preconditioning agents prior to the conduct of clinical studies in humans. Drug development is difficult, expensive, and relies on multifactorial analysis of data from diverse disciplines. Importantly, there is no single path for the preclinical development of a novel therapeutic and no proven strategy to ensure success in clinical translation. Rather, the conduct of a diverse array of robust preclinical studies reduces the risk of clinical failure by varying degrees depending upon the relevance of preclinical models and drug pharmacology to humans. A strong sense of urgency and high tolerance of failure are often required to achieve success in the development of novel treatment paradigms for complex human conditions.

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Introduction

The typical process for the evaluation of a novel preconditioning treatment includes in vitro cell-based assays and in vivo animal models of ischemia–reperfusion (I/R) injury or stroke. The breadth of studies recommended prior to clinical evaluation of novel agents depends upon a number of factors including: (1) the nature of the treatment, (2) the identity or location of the therapeutic target (e.g., receptor distribution and organ specificity), (3) the intended clinical indication (e.g., acute or chronic condition, comorbidities, age, sex, and species relevance), and finally, (4) the risk/benefit profile. Several clinical applications for preconditioning agents have been proposed which involve treating patients at elevated risk of ischemic brain injury. The risk of treatment versus the potential clinical benefit varies for each patient population and may significantly influence the clinical development path. Preconditioning treatments can involve devices, therapeutic modalities, or drug treatments, and each of these pose different challenges for clinical development. The target of a given therapy could reside in the peripheral circulation in the central nervous system (CNS) or both, further complicating preclinical modeling.

The selection of preclinical model depends on the particular aspect of human physiology that is relevant to the proposed treatment. For example, a therapeutic treatment that mitigates inflammation resulting from I/R injury would necessitate the use of models that recapitulate inflammatory processes present in afflicted humans. The primary objective

of early stages of development of a therapeutic approach is to validate direct target activity. Therefore, simplistic in vitro models with direct target activity are often employed, rather than a complex disease model. Upon verification of on-target potency, later stage preclinical studies typically involve intricate cell-based systems or in vivo modeling. In cases where the target remains unclear, multiple models (in vitro and in vivo) may be necessary to assess the potential for treating humans. Basic research continues to identify mechanisms of I/R injury. Most therapeutic strategies, particularly targeted agents, fail to address all aspects of I/R injury (e.g., inflammation and excitotoxicity), and thus, some models may demonstrate better drug efficacy than others. Such disparities in drug performance could uncover new insights regarding mechanisms of action or may emphasize a particular clinical development path. Thus, it is prudent to test a drug candidate in multiple systems and across multiple species to maximize the predictive value regarding in vivo efficacy or safety in humans.

Rigorous study design and transparent reporting are essential for the advancement of promising agents for clinical study. Such considerations were reflected in a set of recommendations made by the Stroke Therapy Academic Industry Round Table (STAIR) [1] as well as in a recent guide released by the National Institute of Neurological Diseases and Stroke (NINDS), a document designed to improve the quality of preclinical and clinical research supported by the institute [2]. Testing putative neuroprotective agents in multiple species, including nonhuman primates (NHPs), prior to the pursuit of clinical studies is thought to be prudent. Preclinical studies using the mouse middle cerebral artery occlusion (MCAO) model and a NHP two-vessel occlusion model of cerebral ischemia in the rhesus macaque have been conducted in order to validate toll-like receptor 9 (TLR9) as a target for preconditioning-induced neuroprotection. Based on an abundance of in vitro and in vivo preclinical efficacy data in both rodents [3, 4] and NHPs [5], TLR9-targeting agents are good candidates for clinical evaluation. One such TLR9 agonist, a CpG oligonucleotide (ODN) optimized for human and NHP stimulation, demonstrates potent efficacy reducing infarct and neurological outcomes by twofold in a NHP two-vessel occlusion model of cerebral ischemia with protection evident for up to 7 days poststroke (unpublished data). Many of the preclinical principles discussed in this article have been instrumental in the advancement of this basic science research discovery to preclinical development and clinical readiness.

Early Preclinical Development Using Cell-Based Assays

A typical first step in the preclinical evaluation of a therapeutic candidate employs in vitro models that closely mimic relevant in vivo cellular responses. The choice of appropriate target cells and physiologically relevant environmental conditions is important. While commercially available immortalized cell lines can be valuable tools, primary cells more accurately reflect lineage-specific properties associated with the in vivo environment. Often, genetically manipulated cells overexpressing the target of interest or a reporter cell line sensitive to target modulation are used for the initial screening to compare the absolute potency of various treatments. Once "hits" are selected, a more physiologically relevant system (e.g., primary cells) is used for the prioritization of clinical candidates. In vitro screening also serves to evaluate, very early on, if there is any apparent toxicity to mammalian cells. Therefore, cell death due to drug treatment alone and efficacy of the drug in the assay are typically observed simultaneously. Several in vitro assay systems that mimic in vivo physiological processes with substantial relevance to I/R injury have been developed.

Modeling I/R injury in the brain involves the isolation and culture of cells derived from the CNS, as they are the typical cellular targets of most therapies. The most common in vitro cell-based assay used to evaluate the potential protective effect of a drug against I/R injury consists of primary cortical cultures derived from the microdissected cortical tissue of late stage embryonic rats or mice. These cultures consist of multiple cell types within one culture comprised of neurons, astrocytes, and microglia. These mixed cultures more accurately reflect the cellular composition of cortical tissue allowing interactions between cells and their secreted products. This is particularly important in screening a potential therapy as individual cell responses and complex crosstalk between cell types can affect the assay outcome. However, although advances have been made in terms of the basic molecular mechanisms underlying neuronal death, clinically effective neuroprotective drugs for treating acute stroke have not yet been discovered [6], leading to the assumption that a singular focus on saving neurons alone might not be sufficient. There is an urgent need to integrate and extend the current studies of neurons to include other cellular components of the neurovascular unit, such as cells comprising the blood–brain barrier (BBB) [7].

The neurovascular unit has shown significant importance to I/R injury and consists of the dynamic interactions between cerebral endothelial cells, astrocytes, neurons, and the extracellular matrix. The concept of the "neurovascular unit" highlights the importance of multiple cell types in both brain injury and neuroprotection [8, 9]. Within the neurovascular unit, the role of cerebral endothelium belonging to the BBB is particularly important, since it actively participates in cerebral functions, both in physiological and pathological conditions [8]. Hence, elucidating the mechanisms of cells signaling within the neurovascular unit may be crucial in the ongoing search for effective therapeutics for CNS conditions including I/R injury or stroke.

Modeling the Blood–Brain Barrier

The BBB is a selective barrier formed by the endothelial cells that line cerebral microvessels [10, 11]. It acts as a 'physical barrier,' since complex tight junctions between adjacent endothelial cells force most molecular traffic to take a transcellular (through the cell) route across the BBB, rather than moving paracellularly through the junctions [12]. More importantly, the BBB protects the brain from fluctuations in ionic composition that can occur after a meal or exercise, which could disturb synaptic and axonal signaling [13]. The barrier helps to keep separate the pools of neurotransmitters and neuroactive agents that act centrally (in the CNS) and peripherally (in the surrounding tissues and blood), so that similar agents can be used in the two systems without 'crosstalk.' Because of its large surface area (~20 m^2 per 1.3 kg brain) and the short diffusion distance between neurons and capillaries, the endothelium plays a prominent role in regulating the brain microenvironment.

There is strong evidence, particularly from studies in cell culture, that astrocytes can modulate many BBB features, leading to stronger tight junctions (physical barrier) [14]; the expression and polarized localization of transporters, including Pgp24 and GLUT1 (transport barrier) [17]; and specialized enzyme systems (metabolic barrier) [10]. More recently, other cell types present at the BBB, including pericytes, perivascular macrophages, and neurons, have also been shown to contribute to barrier induction [15–18]. The converse induction, in which brain endothelium enhances the growth and differentiation of associated astrocytes, has also been demonstrated [19, 20]. All of these features and cellular interactions need to be taken into account in the attempt to mimic the in vivo BBB using an in vitro cell-based system.

Challenges for the Development of CNS-Targeted Therapeutics

The existence of the BBB and the complex interactions that act to regulate it pose a challenge for therapeutic targeting of the CNS for stroke. Certain drugs will need to cross this barrier to be effective. Tight junctions are responsible for the severe restriction of the paracellular diffusional pathway between the endothelial cells to ions and other polar solutes and effectively block penetration of macromolecules by this route. The impediment to ion movement results in the high (~1,800 Ωcm^2) in vivo *trans*-endothelial electrical resistance (TEER) across the BBB [21], emphasizing the extreme effectiveness of the tight junctions. In addition, the presence of specific transport systems on the luminal and abluminal membranes regulates the transcellular traffic of small hydrophilic molecules, which provides a selective 'transport barrier,' permitting or facilitating the entry of required nutrients and excluding or effluxing potentially harmful compounds [22] including potential therapeutic drugs.

Finally, ectoenzymes such as peptidases and nucleotidases provide a 'metabolic barrier' capable of metabolizing peptides and ATP, respectively, whereas intracellular enzymes such as monoamine oxidase and cytochrome P450 can inactivate many neuroactive and toxic compounds [23]. Large hydrophilic molecules such as peptides and proteins are generally excluded, unless they can be transferred by specific receptor-mediated transcytosis or by the less specific adsorptive-mediated transcytosis [24]. However, the brain endothelium has a much lower degree of endocytosis/transcytosis activity than does the peripheral endothelium, which contributes to the transport-barrier property of the BBB. Hence, the term 'blood–brain barrier' covers a range of passive and active features of the brain endothelium. As the tight junctions severely restrict entry of hydrophilic drugs and there is limited penetration of larger molecules such as peptides, strategies for drug delivery to the CNS must take these features into account. On the other hand, some drugs may exert their protective effects by acting on non-CNS-resident cells. Therapies targeting cells outside of the CNS may pose fewer development challenges because response to treatment can typically be measured in the peripheral circulation.

Predicting BBB Drug Permeability

Predictions regarding drug permeability across the BBB are made using in silico, in vitro, and in vivo methods and these methods are typically employed in this order of priority. In silico methods are rapid but there is an obvious risk that such predictions are inaccurate. The accuracy of in silico models depends upon the quality and amount of empirical data available for generating the model, and thus, predictions continually improve as the composition and size of the datasets increase. Low cost in vitro methods to measure BBB permeability with good reproducibility have also been developed to screen for drugs with a passive transport mechanism [25]. In vivo methods for testing BBB permeability of drugs while offering a more direct measure with greater importance in preclinical decision-making, suffer from cost and time constraints. Ultimately, all of these methods carry risk and model appropriateness will be dictated by the degree of understanding surrounding the mechanism of action, relevance to the process to be modeled, statistical robustness provided by replicate measurements, and range and distribution of measured data [25].

The BBB as a Therapeutic Target

The establishment of an in vitro BBB model allows for late-stage screening of drugs, not only for their ability to cross

the BBB but also for direct effects on the stabilization of key in vivo BBB features. The first in vitro BBB filter model was introduced in the early 1980s [26]. The first studies used monocultures [26, 27], which led to the discovery of phenotypic loss, a condition overcome by coculture with glial cells [28]. Currently, cocultures comprised of brain endothelial cells and glia are commonly used as in vitro reconstituted BBB models.

Despite the clear advancement in BBB modeling provided by the use of abluminal endothelial–glial cocultures, these "static" systems lack the ability to allow for endothelial exposure to physiological shear stress (SS), which is a frictional force generated by the exposure of the apical membrane of the endothelial cells to flow. Given the unique vascular modulatory role of shear forces, as well as that of abluminal astrocytes (and perhaps pericytes), it is not surprising that attempts have been made to develop more sophisticated dynamic (flow-based) coculture BBB models. One of these dynamic in vitro BBB (DIV-BBB) models uses hollow fibers that act as artificial blood vessels where primary cultures or cell lines of animal or human brain microvascular endothelial cells can be cultured surrounded by an extracapillary space (the brain side) cultured with astrocytes. These fibers are then exposed to quasiphysiological pulsatile laminar SS. This model more closely resembles the in situ BBB both functionally and anatomically [29].

Modeling I/R Injury in Cell-Based Systems

Blockage of blood flow in ischemia deprives tissue of nutrients and oxygen. Cells of any type (e.g., cell lines, primary endothelial, neuronal, or glial cells) can be exposed to an environment that mimics ischemia by depriving them of oxygen and glucose (oxygen–glucose deprivation, OGD). The ability of an agent to protect against cell death from OGD can be evaluated by quantifying dead cells as a proportion of live cells in the culture system. OGD can be administered prior to or following treatment with potential therapeutics to evaluate the cytoprotective capacity in the setting of ischemia.

In vitro BBB models can also be used to study the protective effects of promising drugs. In addition to cell death, both functional (e.g., TEER and endothelial permeability) and structural (e.g., tight junction proteins) outcomes can be measured using these systems. Susceptibility of brain endothelial cells to hypoxia differs significantly depending upon time, culture and treatment conditions, and validity of the models used. Hypoxia (1.5–24 h) induces a drop in TEER and an increase in endothelial permeability in both endothelial monolayers [30–33] and coculture systems [34–36]. In all models tested, hypoxia combined with glucose deprivation resulted in a much faster (2–4 h) increase in endothelial permeability for sucrose, inulin, apotransferrin, and albumin than in hypoxia alone [37, 38].

The role of glial factors in mediating the effect of hypoxia is controversial. Some factors can worsen the ischemia-induced increase in endothelial permeability [37] or have a protective role attenuating the hypoxia-induced increase in permeability [31, 39]. In vitro hypoxic conditions affected the localization of the tight junction protein claudin-5 to the plasma membrane and its overall protein expression level [40]. Ex vivo examination of cerebral ischemia identified a decrease in occludin and ZO-1 after microsphere-induced cerebral embolism [41]. In another in vitro assessment, hypoxia increased paracellular permeability along with the disruption of occludin, ZO-1, and ZO-2 membrane localization [33]. Thus, the observed increases in paracellular permeability generally correlate with a loss of TJ protein localization and/or expression along the cellular membrane.

A good example of preclinical studies using these cell-based assays involves the evaluation of the protective effect of antecedent treatment with TLR agonists. The cell types represented in primary cortical cell cultures (i.e., astrocytes, neurons, and microglia) express TLRs, and thus, all are potential target populations in this assay. The TLR expression profile on each cell type is variable and activation of one cell type ultimately affects others in the culture. In our studies, primary cortical cells were treated with a TLR agonist 1 day prior to exposure to 3 h of OGD. Pretreatment with various TLR agonists effectively protected primary cortical cells from OGD-induced cell death [4, 42, 43]. To our knowledge, none of the TLR agonists tested to date have demonstrated apparent toxicities at effective preconditioning doses.

Another interesting aspect of TLR agonist preconditioning is its effect in non-neuronal systems. Using an in vitro BBB model consisting of a coculture of primary murine brain microvessel endothelial cells (BMEC) and primary mixed astrocytes and microglia cells, we showed for the first time that preconditioning with a TLR ligand, polyinosinic polycytidylic acid (poly-ICLC), attenuates OGD-induced BBB dysfunction (e.g., TEER and permeability) and integrity (e.g., preservation of tight junctions) of the BBB endothelial cells [44]. In addition, our study implicated IFNβ as a key player in the protective effect induced by poly-ICLC preconditioning on the BBB [44]. The significance of these findings was highlighted by in vivo studies, indicating that poly-ICLC preconditioning depends on type I IFN signaling to protect the brain against ischemic injury [44].

Limitations of In Vitro Modeling

While in vitro testing of cell-based assays is an informative first step, a major limitation of the in vitro paradigm for drug

screening is that it is a closed system. In this system, no other tissues or organs are affected by the administration of the drug, which could dramatically alter the therapeutic outcome and could also mask systemic drug toxicities or inferior pharmacology. Also, depending upon the specific drug target (e.g., peripheral circulation, CNS, and microvascular endothelial cells), in vitro systems may not be practical or informative as to the potential drug efficacy in animal models. In the age of "targephilia" or emphasis on target-based drug discovery, a growing challenge will be the development of appropriate model systems with physiological relevance.

None of the assays proposed above can accurately reflect all cellular interactions or the dynamic milieu of secreted proteins active in the whole organisms following treatment with a drug or in the context of injury. For instance, when the brain becomes injured, there are many changes that take place in the organism. Modeling changes occurring in distant organs or systems could be of paramount importance for assessing the effectiveness of a given treatment approach targeting a given protein or pathway. In addition, the behavior of a drug in the whole animal may be different than in isolated cells. The in vivo metabolism of a drug could generate metabolites of unknown activity or toxicity, a feature that should be accounted for early in preclinical development.

Positive results in in vitro screening assays should be interpreted with caution. The absolute in vitro potency of a treatment is often unjustifiably used for prioritization or selection of clinical candidates. However, these results are not necessarily indicative of in vivo potency or efficacy in animal models due to the potential for variability of in vivo pharmacology. For instance, a molecule having twofold greater bioavailability may be a more attractive clinical candidate, despite having 25–50 % less potency in a cell-based system. The rationale for this scenario is that getting more drug in the bloodstream for longer periods of time ultimately translates to better efficacy despite less on-target potency.

Additionally, protection in the OGD assay using cortical cultures does not provide definitive proof that a drug is acting in the brain when administered to animals. A central effect could merely be coincidental in the case of agents found not to cross the BBB in vivo following systemic administration. For example, many TLR agonists that are not thought to readily cross the BBB show potent neuroprotection in vivo following systemic administration while also showing potent efficacy in isolated neuronal cultures subjected to OGD [42]. Recent data from our laboratory using TLR9 chimeric mice revealed that neuroprotection depends upon target expression in the brain and also in parenchymal cells [45], suggesting more than a central acting mechanism. Clearly, in vivo models are exceedingly beneficial as they begin to address these major deficits inherent to in vitro systems and they more effectively reveal the mechanism of action, safety, and pharmacology of a novel therapeutics, attributes of significant importance for clinical application.

Modeling Ischemic Brain Injury in Animals

Many strategies are used to develop novel therapeutics for human conditions. Most approaches use animal models. Regulatory agencies responsible for drug approval require that critical preclinical data involve studies in mammals. As such, animal studies act to reduce late-stage attrition due to lack of drug efficacy or unforeseen safety issues and play an important role in clinical trial design. Animal testing is often used for risk assessment (i.e., determining the risk of taking a drug to market for a particular clinical indication) even when the clinical predictive power of a given model is unproven. This is especially true with respect to safety, as lessons learned from studies in multiple species and in the presence of disease pathology can dramatically reduce the risk of administering drugs at doses that may be unsafe in humans. First-in-human trials are typically conducted using doses that are projected based upon preclinical animal modeling.

Many characteristics of human ischemic brain injury can be modeled in experimental animals [46, 47]. The most common type of human stroke is focal ischemia due to occlusion of the middle cerebral artery (MCA) [47], which results in little or no blood flow to some regions of the brain. The early phase of injury involves the development of a core zone of severe ischemia that represents irreversibly injured tissue. Late phase injury develops in the ischemic penumbra, an area adjacent to the core infarct zone that is at risk of eventual infarction if blood flow is not quickly restored.

The penumbra remains a major target for acute therapies as it is considered a salvageable region of brain tissue most likely to be protected by pharmacological, thrombolytic, or mechanical (recanalization) intervention. Thus, it is ideal that cerebral ischemia modeled in animals contains a core and penumbra infarct zone to obtain physiological relevance with regard to drug targeting of these distinct features of stroke-induced brain damage. In the context of preconditioning, the resulting size of the core infarct zone may also be reduced due to protection offered by tolerance mechanisms. This would be distinct from acute therapies, which have minimal opportunity to affect the size of the core infarct area. Preconditioning may also increase the time to treat with other acute therapies, thus widening their therapeutic window and giving new life to many marginally effective or failed stroke drugs.

Modeling in Rodents

Although several rodent models of focal cerebral ischemia have been developed [48], focal ischemia is often modeled

in the mouse by using transient occlusion of the middle cerebral artery (tMCAO) by insertion of a silicone-coated surgical suture to unilaterally block blood flow and cause a progression to ischemic brain damage. This model is transient in nature and creates a quantifiable infarct zone comprised of a core and penumbra and neurological deficits relevant to that seen in the human condition [49]. Reproducible infarcts can be achieved by controlling the duration of ischemia. Mouse models routinely provide efficient insight into the understanding of the pathophysiology of I/R injury and potential for drug efficacy. These models provide a platform for optimization of lead compounds based on overall efficacy and on-target potency. They also provide a first look at in vivo drug pharmacokinetics (PK), biological activity, and potential toxicities. Despite their utility, in vivo rodent models are limited in their ability to faithfully recapitulate human disease.

Preclinical Studies in Nonhuman Primates

Many recent efforts to translate preclinical acute neuroprotective strategies from rodents to humans have been disappointing [50]. The lack of congruency observed may be due to species differences including vast differences in brain tissue structure, composition, and tolerance to ischemia, as well as variation in drug–target interaction, target expression, or distribution. Additional species-dependent properties with regard to in vivo PK, pharmacodynamics (PD), or precise mechanism of action may also contribute to failures. These limitations have prompted the development and implementation of NHP models for the evaluation of stroke therapeutics.

NHP models offer significant advantages for preclinical testing of candidate neuroprotective agents due to their close phylogenetic relationship to humans with similar neuroanatomy, vasculature [51], and gyroencephalic brain morphology. Humans and NHPs have a similar ratio of gray to white matter [52], yielding similar thresholds to ischemic injury [53]. These similarities suggest shared mechanisms of ischemic injury and endogenous neuroprotection may exist. Infarct volumes in NHP, such as the rhesus macaque, have been effectively quantified using magnetic resonance imaging (MRI) [54]. Novel user-independent MRI analysis methods are currently being validated, methods that could reduce bias and speed data analysis of large cumbersome preclinical datasets (data not shown).

In addition, the complex behavior of these animals allows for a better assessment of neurological or behavioral deficits than that of rodent models. Hemiparesis is commonly observed following stroke affecting the upper limb and face primarily, although a wide range of motor deficits can be seen in both humans and experimental animals. Anecdotal observations from our laboratory in recent efficacy studies suggest that improvement over time of proximal and distal limb function can be observed in this model. This may be an important outcome measure for consideration in studies with acute or preconditioning paradigms where longer durations of observation poststroke are examined (~14–28 days). The use of clinically meaningful neurological scales and actimetry [55] monitoring of physical activity in our NHP models provide functional outcome measures of cerebral ischemia that more accurately reflect the human condition in stroke. Finally, studies performed in NHPs allow for better feasibility assessment. Studies in NHP can provide PK and PD profiles for drugs, as well as information regarding efficacious and toxic dose ranges, data more applicable to human subjects. As such, selective NHP studies offer an important translational bridge to clinical studies by providing a pragmatic model for target validation, biomarker identification, testing efficacy, and optimization of novel therapeutic strategies prior to clinical studies in man.

In the NHP, artery occlusion in the brain is accomplished via the use of aneurysm clips, thrombus, or by embolization. Most models of stroke in NHP require significant neurosurgical skill to implement effectively. MCAO models in monkeys often produce infarcts that vary in location and size [56, 57]. Longer durations of ischemia are necessary to induce damage beyond the basal ganglia due to collateral flow to the region from the anterior cerebral arteries. Unfortunately, the durations of occlusion that cause cortical damage often produce cortical and subcortical damage associated with high morbidity and mortality [58, 59]. Our group has developed a less invasive model of ischemic stroke in male rhesus macaques [54]. Using this *trans*-orbital reversible two-vessel occlusion model, we have achieved an infarct that is primarily cortical and mimics the injury observed in humans resulting from occlusion of the MCA. The resulting volume of infarcted brain tissue is typically ~8 % of the whole brain or ~22 % of ipsilateral hemisphere and ~45 % of total cortical area in Chinese adult male rhesus macaques (aged 6–12 years and 6–12 kg body weight) after a 60-min MCA/ACA occlusion. This model is particularly well adapted to preclinical study, as there is less subcortical or striatal involvement, less morbidity, and reduced variability. While the volume of the infarct typically correlates well with neurological and histopathological outcomes in untreated animals [54], the possibility exists that these parameters will not always correlate depending upon the drug target. In the case of TLR9 agonist-induced preconditioning, the infarct volume and neurological findings indeed correlated in our drug-treated animals in the rhesus model, similar to that seen in controls (unpublished data).

Testing Novel Preconditioning Paradigms in Animals

Key components of in vivo preclinical evaluation using stroke models in animals include determination of the dose,

time window of effectiveness, and route of administration. The use of in vivo models for this line of investigation is crucial because they incorporate drug behavior and the systemic response of all tissues. The in vivo system also accounts for the role of the BBB in drug distribution and efficacy, dose route requirements, and therapeutic window. For example, systemic administration of CpG ODN will activate TLR9 receptors found on hematopoietic cells such as neutrophils and dendritic cells (DC). The introduction of these hematopoietic cell responses (i.e., cellular activation or secretion of cytokines) in the context of in vivo treatment could alter the response to the drug or be responsible for additional efficacy or toxicity.

Due to the mechanisms involved in preconditioning, a therapeutic is not required to be present at the time of the insult to exert neuroprotective effects. For example, to evaluate the effects of antecedent TLR stimulation in mice or monkeys, TLR agonists are administered 3 days prior to occlusion resulting in dose- and time-dependent neuroprotection. The protective time window for TLR agonists generally extends from 1 to 3 days prior to the tMCAO, a highly feasible time window for translation to clinical studies. This protective window falls within the timeframe that surgery patients receive preop appointments.

Route of drug administration is an important consideration since the effective route needs to be convenient and simple to implement with minimal patient discomfort. Drugs administered intranasally (IN) are rapidly absorbed and transported through the cell and capillary-rich mucosal surface by olfactory neurons and the cerebral spinal fluid [60]. Subcutaneous (SC) injection delivers the drug just beneath the skin and demonstrates robust and predictable absorption through the capillaries. These systems are advantageous due to their typically high drug bioavailability.

Additional late-stage preclinical evaluations may involve the use of animals with specific comorbidities relevant to the human clinical population such as models of aging, diabetes, atherosclerosis, or other metabolic syndromes. Many of these complex disease models vary in their ability to mimic the phenotypes present in high-risk patients and many have posed challenges for drug development for their primary indication. The use of aged animals has been recommended to account for comorbidities and differences in aged animal physiology; however, rodents and even NHPs are likely inadequate to recapitulate hypertension, atherosclerosis, and other common stroke comorbidities. There are many parallels between mouse and human aging; yet the marked difference in lifespan and metabolic stability pose intrinsic limitations to the use of aged mice. The use of complicated animal models combined with a complex condition like brain ischemia or stroke could produce erroneous results without careful model development and characterization. It could take decades to develop robust preclinical models and even longer to validate them as relevant models for clinical development. While this effort should be pursued, it should not preclude the continued exploration of novel agents in clinical studies.

Principles of Preclinical Study Conduct

The goal of preclinical research is to identify candidate drugs that reproducibly give credible and predictable data with regard to biological activity, a feature indicative of druglike properties. As such, rigorous study design, conduct, and reporting are hallmarks of good preclinical development programs. Regardless of whether the study is being performed in an academic setting, at a company, or by a contract research organization (CRO), the detailed parameters of a formal preclinical study should be determined a priori via a formal written study protocol including data processing and statistical analysis approaches. This practice limits any unintended improvisation or bias with regard to methods of data collection, blinding, study execution, data review, exclusion criteria, and methods for statistical analysis and reporting. Preclinical efficacy studies should not be mistaken for target validation or mechanistic studies, as their purpose is very different. Their purpose is to evaluate the robustness of the agent, as the ability to modulate the target and efficacy should already be established from preliminary studies performed in a research setting.

Blinded evaluation is a critical component of preclinical studies. Human bias is unpredictable and known to impact the outcome of research studies. Bias can be minimized by the coding of study subjects and samples and by blinded observation. Additionally, any necessary data exclusions should be determined prior to treatment identity being revealed in order to ensure an unbiased evaluation. Moreover, the number of animals required for each study should be determined using power analysis prior to study initiation. The analysis of data using appropriate statistics for the number of groups included in the study is exceedingly important, as the variance across all groups can impact the significance of the findings. Appropriate randomization is particularly important for stroke studies since the time-consuming nature of the model requires strokes to be conducted on several animals per day over multiple study days increasing the potential for variability even with a single surgeon. The inclusion of all relevant controls in each experiment is important to adequately compare one or more therapeutics across different preclinical studies. Under circumstances where a given candidate therapeutic is exceedingly expensive or drug quantities are limited, specific power calculations can be employed that serve to limit the number of drug treated animals while still achieving optimal power by increasing the number of controls.

Again, all pertinent study parameters should be carefully determined prior to preclinical study initiation. Once a formal drug study has officially begun, none of the study parameters should be altered without specific justification. Deviations will inevitably occur; however, all deviations, large or small, from the original study design should be carefully documented to allow for potential exclusions prior to the analysis of the final dataset. Strict adherence to these principles remains a cornerstone of industry-style preclinical development practices where many systems are employed to ensure compliance. These practices, while seemingly daunting to implement in an academic environment, are known to heighten the reproducibility of study findings and mitigate risk for clinical development. Often the best approach is to employ a CRO to conduct studies to provide an independent assessment, which serves to strengthen confidence in the preclinical program and further reduce risk. This is not an uncommon practice even for pharmaceutical industry-run programs intending to license their technologies to larger companies, despite the fact that they have internal resources to conduct similar studies.

PK/PD Modeling

One of the most important aspects of preclinical evaluation is the establishment of a dose–response relationship, a feature that defines the therapeutic window with respect to dose and potential toxicity of the treatment. Plasma drug levels and/or bioactivity measures reflective of treatment-dependent changes in biological functions should be included in all preclinical efficacy studies. The practice of profiling in vivo drug exposures and bioactivity is referred to as PK/PD modeling [61]. PK/PD information is often used in preclinical settings to validate novel targets or drug mechanisms of action, and ultimately to improve later clinical translation. While it may seem obvious that greater drug exposure leads to greater efficacy and potential for side effects, this is not always the case. In the context of preconditioning with TLR agonists or other paradigms, it is well known that doses too high or too low are ineffective and rarely detrimental within a reasonable dose range. The generation of a dose–response curve (nonmonotonic) with inverted U shape is a hallmark of preconditioning agents. For example, studies in mice using pharmacological preconditioning agents often result in a dose–response curve reflective of the fact that very low and very high doses are ineffective. This response fits with the assertion that preconditioning requires a small dose of an otherwise harmful stimulus to achieve protection against a subsequent injury. Therapeutics with this type of dose–response curve can be challenging for clinical development without the use of validated biological markers (biomarkers) that serve to indicate the achievement of a therapeutic dose.

Importance of Biomarkers for Clinical Translation

Biomarkers, as defined by Hulka et al. [62], are "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids." Biomarker identification can take considerable time, so the process should begin in early preclinical studies in rodents and should factor in feasibility of clinical use. The typical goal of a biomarker in early drug development is to validate that a drug hits the intended target, modulation of that target alters a disease phenotype, and this alteration leads to a long-term clinical improvement. The most useful biomarkers for clinical development are those that significantly correlate with efficacy in a dose-related manner.

Peripheral blood-derived biomarkers (e.g., changes in cellular responses, cellular distribution, or plasma proteins) can and should be developed when a treatment is known to modulate biological processes that can be detected in the blood or other fluid (e.g., saliva or urine). Biomarkers in the blood that correlate with brain biomarkers could be invaluable in clinical development of preconditioning agents, as they serve to predict the establishment of a neuroprotected state in patients prior to putting them at risk of ischemia. Drug-induced biomarkers present in the brain cannot be measured readily in humans, although they are useful when measured in animal models to identify surrogate blood markers. For example, CpG ODN preconditioning agents that stimulate TLR9 elicit predictable and dose-dependent systemic and brain inflammatory responses representing drug-induced biomarkers including systemic cytokines/chemokines and cellular activation manifested as cell surface protein expression (e.g., CD69, B7-2), as well as brain cytokine/chemokine gene expression (e.g., L-1beta, IL-6, IL-12, TNFalpha, MIP-1alpha, and/or MIP-1beta) [63–66]. Systemic treatment with CpG ODNs robustly induced dose-dependent induction of interferon associated protein-10 (IP-10) in humans [65] and rhesus macaques [67] with levels persisting for several days. IP-10/CXCL10, as well as the antiviral cytokine 2′,5′-oligoadenylate synthetase (OAS), have fast become important biomarkers indicative of in vivo immune activation secondary to TLR activation in clinical and preclinical studies [65]. In addition, biomarkers in the brain that correlate with TLR9-induced neuroprotection in animal models have been identified including *tumor necrosis factor-alpha* (*TNF*) gene expression [45]. In the ideal scenario, peripheral biomarkers of drug activity (e.g., IL-6 and IP-10) would be present in plasma following the administration of efficacious doses of drug to experimental animals. These biomarkers can be useful even if no causation can be established as they can reflect duration and potency of drug activity. Systemic biomarkers in the blood or other accessible tissues from humans obviate the need for access to brain tissue or the implementation imaging approaches that could be too costly or risk-associated.

Biomarkers also provide a means for retrospective data analysis in the event of failure or success in preclinical or clinical settings. In the acute setting of stroke, proof of concept clinical studies have been negatively affected by the heterogeneity of both the patient population, in general, and their spectrum of stroke severity. The monitoring of biomarkers can account for unanticipated variability in PK or drug responsiveness among patients, particularly in the diseased state. Subjects with poor drug response regardless of the reason (e.g., genetic, food consumption, timing, stress, and disease stage) can often be accounted for by using biomarker analysis. We postulate that biomarkers exist in NHP plasma following CpG ODN dosing and these biomarkers correlate with the extent of neuroprotection following ischemic injury. We predict there will be a correlation between efficacy in the brain and some systemic blood-derived biomarker reflective of drug bioactivity acutely following CpG ODN treatment prior to I/R injury. Studies are ongoing to identify predictive biomarkers in the hopes of using this strategy in clinical trials.

The fact that dose–response studies in mice have revealed an inverted U-shape curve argues that PK/PD analysis may prove even more applicable in this case. Identifying whether the degree of peripheral drug response correlates with neuroprotection will be important for risk assessment, since historical industry bias dictates that unusual dose–response curves denote higher risk of clinical failure. For preconditioning, the shape of the dose–response curve is likely reflective of the mechanism involved in establishing neuroprotection and not the typical drug parameters known to be responsible for heightened risk. In the context of genetically diverse populations (humans and monkeys), often an inverted U-shaped PK/PD curve can also be obtained as an artifact when patients less sensitive to the drug need higher doses to elicit a response. It may appear as though the response decreases after a certain dose in a heterogeneous human population; however, these data could instead reflect differences in sensitivity to the drug. This feature is often not perceived in preclinical studies due to the use of genetically identical animals. A robust PK/PD strategy obviously does not guarantee successful clinical dose extrapolation, but it is a useful option when the target or mechanism of a therapy is poorly established, when a drug mechanism is unprecedented, when differences in drug responsiveness are anticipated, or when direct access to the target tissue is prohibited.

The reproducibility of the agent in preclinical models and across laboratories has become a key factor for consideration. Demonstrating robust efficacy in one or more related preclinical models often indicates that an agent demonstrates reasonable PK and is either broadly active or active against a proximal mechanism of a common disease pathway, demonstrating good druglike properties. Agents that periodically fail to show efficacy in animal models may have complex PK or require precise model parameters, both of which could pose challenges for clinical development but also could simply reflect certain limitations of a model system. Periodic failure in preclinical models is not uncommon and does not invalidate a therapeutic target or agent but rather should prompt a search for better models with greater target-related pathology or additional candidates that modulate the target more reliably.

Translating TLR9 Agonists

Stimulation of TLRs, a family of evolutionarily conserved receptors traditionally associated with regulation of innate immunity, elicits robust neuroprotection typically 3 days following systemic administration [68]. While the precise molecular mechanisms governing TLR-induced neuroprotection are unclear, it is currently believed to represent a form of “tolerance” [69] often described as a hyporesponsive state induced by low-level activation of TLR signaling [70]. Several TLR agonists recognized by TLR2, TLR3, TLR4, TLR7, or TLR9 have been shown to protect against ischemic injury in rodent stroke models [4, 43, 71–73], enhancing the validity to this therapeutic approach. Specifically, preconditioning with these TLR agonists 3 days prior to stroke resulted in reduced infarct volumes. Efficacy using TLR agonist preconditioning is generally correlated with the early induction of proinflammatory mediators in the brain and systemic circulation within hours of systemic administration followed by the induction of interferon-related genes after I/R injury. While the precise mechanisms are unknown, these factors represent potentially important biomarkers of neuroprotection for use in clinical development of TLR-mediated preconditioning agents.

One such promising clinical therapeutic agent is unmethylated CpG ODN, a TLR9 agonist that has demonstrated the ability to cause cross-tolerance of TLR4 and TLR2 [74] resulting in a dampening of detrimental endogenous ligand signals typically induced by I/R injury. Recent unpublished results from our laboratory show reproducible efficacy following CpG ODN preconditioning in our NHP stroke model. This novel CpG ODN preconditioning agent results in twofold reductions in infarct size and neurological deficits with no apparent toxicities acutely following treatment or in the context of stroke at doses approximately fivefold higher than the efficacious dose. A preponderance of preclinical evidence indicates that TLR signaling is a target pathway that can be exploited to induce a neuroprotected state in high-risk patients. CpG ODNs are candidates for repurposing as they have already exhibited in human studies the following features: (1) desired biological activity without apparent toxicity, (2) reasonable PK and ADME (absorption,

distribution, metabolism, and excretion) attributes, and (3) known dose-limiting toxicities that, when present, are consistent with mechanism of action. Importantly, antisense ODN drugs not containing CpG motifs have been approved by the United States Food and Drug Administration (US FDA), establishing a regulatory pathway for this general class of drugs [75]. Species-specific differences in the immune response to synthetic phosphorothioate-modified CpG ODNs exist due to different receptor distribution and sequence specificity [76] further justifying the need for validation in NHPs prior to initiation of human studies. Using the above principles, several CpG ODNs have been evaluated in our laboratory to determine their potential to precondition against cerebral I/R injury in multiple species [4, 5]. Preclinical studies in mouse and, more recently, in monkey have revealed that low doses of CpG ODNs have the potential to provide reproducible neuroprotection when given ~3 days prior to I/R injury.

Current Clinical Development Challenges

Beyond the use of these techniques for new target discovery, preconditioning remains a promising therapeutic strategy for an array of conditions involving human ischemic injury. A key component that remains to be determined is the identification of the most informative clinical population and outcome measures to provide an initial testing path for a given preconditioning paradigm. For instance, preconditioning patients prior to surgeries that carry high risk of brain or peripheral organ ischemia may provide significant clinical benefit. It is clear that several clinical subpopulations are at high-risk of ischemic injury to the brain and other organs; however, many of these populations also have comorbidities that can complicate clinical development of novel therapeutics and necessitate the use of large numbers of patients per trial. Nevertheless, proof of concept trials are needed to propel pharmacological preconditioning therapies to the forefront.

It is important to emphasize that unlike acute stroke treatments, preconditioning paradigms have not yet failed in clinical development. Preconditioning is a preventative treatment designed to alter the response of the tissue to subsequent ischemia or injury and thus, is likely mechanistically very different from agents that are designed to target already injured tissue. Preconditioning as a therapeutic strategy takes advantage of a circumstance where a prophylactic treatment can mitigate a harmful response before it occurs. We feel that this circumstance carries enormous potential since some brain tissue in the context of ischemic injury will inevitably be unsalvageable due to treatment delay despite even the most effective acute therapies. It is also possible that preconditioning could salvage some failed acute stroke therapies by providing an extended timeframe within which acute treatment can be administered to patients suffering I/R injury.

Another important challenge for the development of novel therapies by academic scientists involve the costs required to file an investigational new drug (IND) application, including the cost to manufacture sufficient drug material and conduct IND-enabling studies (i.e., chemical characterization, analytical and formulation development, toxicology, pharmacology, and stability studies). Funding opportunities are available for various aspects of the preclinical and clinical development although to transition from one to the other requires a substantial gap in timing. Lastly, efficient development of novel drug substances requires diverse skills in many fields of study, including but not limited to formulations; chemistry; analytical assay development; scale-up manufacturing; pharmacology; absorption, distribution, metabolism, excretion (ADME); molecular biology; toxicology; immunotoxicology; and clinical medicine, as well as substantial experience in FDA guidelines regarding IND-enabling studies. In an effort to support these activities, NINDS has developed The Blueprint Neurotherapeutics Program offering guidance and support for these often complicated pre-IND activities.

Clinical Populations at Predictable Risk of Ischemic Injury

The use of antecedent therapy to mitigate I/R injury is a promising modality for patient populations at high risk of experiencing ischemia to the brain or other organs, such as those with procedurally-induced strokes or clinically silent ischemic lesions. Clinical populations being considered for first-in-human trials with novel preconditioners include patients undergoing cardiovascular and cerebrovascular procedures. Historically, clinical studies have reported that as many as 6 % of patients undergoing cardiac or vascular surgery, including cardiac bypass grafting (CABG), cardiac valve replacement, carotid endarterectomy, aortic repair, peripheral vascular surgery, and resection of head and neck tumors, will suffer from a frank stroke during or after surgery [77–79]. More importantly, as a result of the advancements in neuroimaging techniques by MRI, specifically diffusion-weighted imaging (DWI), a previously underappreciated incidence of surgery-related ischemic events are known occur in patients undergoing cerebrovascular and cardiovascular procedures [80, 81]. Depending upon the study examined, up to 71 % of patients undergoing endovascular repair for aneurysms demonstrated ischemic lesions following the procedure [82, 83], and lesions were found in 50 % following carotid stenting [84], 22 % after AVM repair [85], and 48 % following open surgical heart valve replacement [86]. A recent study just completed found that 78 % of patients demonstrate ischemic lesions after CABG and 47 % of patients showed evidence of MRI-

detected BBB disruption in this same study [87]. Furthermore, studies of interventional and diagnostic cerebral angiography (CAG), a procedure commonly performed in at-risk patients is thought to carry risk of ischemic injury. One study showed that 43 % of acute stroke patients demonstrated new DWI lesions following CAG as compared to 31 % of stroke patients not subject to this procedure [88].

Importantly, the risk of ischemic injury is likely to continue to increase as more advanced imaging is developed and more complicated surgery and diagnostic procedures are implemented in patients with few options. For example, up to 90 % of patients undergoing a newly approved procedure for *trans*-catheter aortic valve implantation (TAVI) demonstrated ischemic brain lesions and ~5 % suffered stroke within the first year [89, 90]. For at-risk patients, prophylactic neuroprotective strategies designed to reduce the damage caused by a subsequent ischemic injury are highly feasible. While the clinical impact of silent lesions is uncertain and somewhat controversial, the existence of brain lesions in these patients is certain. Their frequency underscores the importance of an approach like preconditioning, a therapy with the potential to limit any potential long-term deleterious effects of ischemia to the brain. Brain I/R is anticipated to occur in these patients and the long-term impact is uncertain, yet patients are only offered acute thrombolytic therapy after the fact to reduce damage, rather than prophylactic protection.

Another important population at heightened risk of brain injury is the US military. Soldiers conducting military efforts in Afghanistan and Iraq are often exposed to explosions from improvised explosive devices (IED) and to extreme environments (e.g., high altitudes and combat), both of which can lead to brain injury including cerebral ischemia and edema. Prophylactic measures could be taken to mitigate the risk of traumatic brain injury (TBI) to military personnel by preconditioning prior to deployment on high-risk missions. The phenomenon of preconditioning in the field of TBI has always received less attention compared to ischemic injury; however, if we look at the literature, one of the first demonstrations of the existence of preconditioning is in a model of TBI. Noble et al. in 1943 [91] showed that rats subjected to small amounts of trauma rapidly acquired a resistance so that they could withstand a degree of trauma otherwise fatal, suggesting that endogenous protective mechanisms also exist in the context of TBI. An early study that combined both ischemia and trauma [92] showed that animals subjected to a transient mild ischemic injury, performed 48 h prior to TBI, had a reduced histological damage at 1 week postinjury, thus showing that a subthreshold ischemic insult could activate endogenous protective pathways following a subsequent TBI.

More recently, the investigation of the endogenous neuroprotective mechanisms in the setting of TBI was studied using heat acclimation (HA), a conserved physiological adaptive process that induces protective effectors. Exposure to 34 °C for 1 month prior to TBI was associated with reduced brain edema at 24 h postinjury, contusion volume at 48 h, and functional sequelae up to 8 days after injury [93–95]. More direct evidence of the existence of preconditioning in the context of TBI has been shown by Longhi et al. [96]. The authors showed that lipopolysaccharide (LPS) preconditioning attenuates the neurobehavioral sequelae and histological damage of TBI. Notably, preconditioning-induced protective effects were robust and persisted for up to 1-month postinjury. In spite of the different pathogenetic pathways involved in stroke and TBI, the present data and results from ischemic preconditioning suggest that the same endogenous protective pathways may be targeted by novel therapeutic strategies in both of these acute brain injuries.

What Indication is Best for First Human Trials with Preconditioners?

A clinical indication that is attractive as a first-in-human trial for CpG ODN preconditioning is patients diagnosed with asymptomatic unruptured aneurysms undergoing endovascular repair procedures. These patients are relatively healthy displaying minimal to no symptoms at the time of diagnosis and have often been identified by chance as having aneurysms. Patients undergoing endovascular repair are at risk for ischemic brain lesions [82, 97, 98]. Silent thromboembolic events related to the use of Guglielmi detachable coils are a common occurrence, despite vigilant technique and systemic anticoagulation. In one study [98], 43 endovascular coiling procedures were examined leading to the identification of 47 ischemic lesions by post-treatment DWI. Most lesions were small (<3 mm), asymptomatic, and located ipsilateral to the aneurysm. The incidence of silent thromboembolic events in another study was 61 % in uncomplicated procedures [82]. A more recent study looked at 185 coiling procedures in aneurysm patients to evaluate the rate of diffusion-positive lesions induced by coiling alone and coiling with Neuroform stenting with or without balloon remodeling [97]. Regardless of the technique used, thromboembolic complications were found in patients with ruptured aneurysms (51 %) and unruptured aneurysms (30 %).

This unique clinical population could provide proof of principle for preconditioning agents in patients with minimal comorbidities. A clinical study of this type was conducted to evaluate neuroprotection by the acute stroke therapeutic, NA-1, in patients that underwent endovascular repair of intracranial aneurysms. In the ENACT phase II clinical trial (NoNO, Inc., Ontario, Canada), the ability of NA-1 to (1) reduce the volume of ischemic embolic strokes,

(2) reduce the number of ischemic embolic strokes, (3) reduce vascular cognitive impairment, and (4) reduce the frequency of large strokes induced by the endovascular procedure were evaluated. Acute treatment with a single dose of NA-1 after endovascular coiling showed a trend for reduced numbers of lesions in patients; work presented by Hill et al. at the International Stroke Conference in February 2012 (New Orleans, LA, USA) and these data prompted the FDA to grant Fast-Track Designation for the reduction of stroke and cognitive impairment in patients undergoing endovascular repair. This clinical development approach may have some advantages in that patients have fewer comorbidities and fewer total patients are required for early phase trials, with only 185 patients included in the ENACT trial. These findings indicate that effective translation of stroke therapeutics (and presumably preconditioners) may benefit from trials in this selected patient population for early mechanistic proof of principle studies [99], prior to the initiation of studies requiring lengthy trials or large numbers of patients (e.g., CABG).

There clearly remains a significant unmet need for safe therapeutic interventions that promote neuroprotection in patients during high-risk procedures and events. Substantial work is needed to decipher the best preclinical and clinical approaches. Importantly, the scientific concepts have been tested rigorously for several decades. More recent evidence shows that powerful endogenous mechanisms of brain tolerance can be induced via TLR stimulation in the primate species [5] and thus, could be exploited for enormous clinical benefit.

Conflict of interest None.

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